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Preliminary data from this study address the first of our four specific research aims: to determine whether blood levels of 1,25-dihydroxyvitamin D, the active metabolite of vitamin D, are lower in women at the time of first diagnosis of breast cancer than in comparable women who do not have breast cancer. To date, we have analyzed the blood of 94 women with breast cancer and 216 women without breast cancer for 1,25-dihydroxyvitamin D. The women with cancer have mean blood levels that are 11% lower than those without cancer. Among white women, this difference is statistically significant at the 0.05 level. Although our number of black subjects is small, there does not seem to be a similar pattern of difference between cases and controls. Our data confirm previously reported differences between blacks and whites in blood levels of 1,25-dihydroxyvitamin D with black women having levels 12% higher than those of white women.

The significance of this work relates to the potential for prevention of breast cancer. Furthermore, there are implications for the development of new therapeutic modalities, and the possibility of better understanding mechanisms of carcinogenesis as we define the role of vitamin D.

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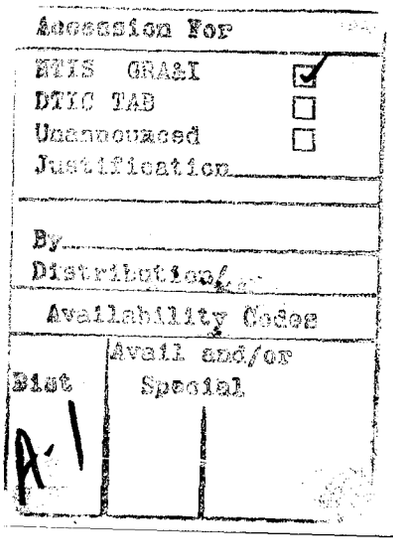

Esther C. Johnson July 14 '95
PI - Signature Date

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INTRODUCTION

Recent work has defined a previously unsuspected involvement of vitamin D in cellular growth and differentiation[1,2]. This recognition has fostered an interest in the investigation of a possible role for vitamin D in carcinogenesis[3]. Several cancers have been evaluated for a relationship between tumor occurrence and low levels of vitamin D. There is a suggestion in the literature that decreased intake of vitamin D may be associated with cancer of the colon[4]; blood levels of vitamin D are lower in African-American and white men with cancer of the prostate than in similar men without prostate cancer[5].

Evidence for the possible relationship between vitamin D and breast cancer is based on several lines of investigation. Ecologic studies generally support a relationship between low levels of sunlight exposure and breast cancer[6,7,8]. A single case-control study in Canada, however, failed to demonstrate an association between low levels of vitamin D, as determined by dietary history, and breast cancer[9]. Studies in rats show an increased incidence of mammary tumors under conditions of low dietary vitamin D (0.05 IU/kcal) and calcium (0.25 mg/kcal)[10]. 1,25-dihydroxy vitamin D ($1,25(OH)_2D$), which is the hormonally active metabolite, displays a growth-inhibitory effect on human breast adenocarcinoma cells in culture irrespective of their sex-steroid dependence[11]. Cultures of the human breast cancer cell line BT-20 demonstrate increased differentiation when exposed to $1,25(OH)_2D$ daily for 8 days[12].

The purpose of our current work is to determine whether there are differences in blood levels of 1,25-dihydroxyvitamin D between women with breast cancer and two control groups of women without breast cancer. We are using archived samples of blood obtained from women in a just-completed case-control study of genetic risk factors for breast cancer. In addition, we are working to modify the immunohistochemical technique for detection of vitamin D receptors (VDR) described for frozen tissue for use in paraffin embedded tissue. If we are successful, we will be able to evaluate the VDR status of tumor tissue from our subjects with breast cancer.

The specific aims of our study are as follows: 1) To determine if blood levels of 25-hydroxy vitamin D and 1,25-dihydroxyvitamin D are lower in women at the time of first diagnosis of breast cancer than in comparable women who do not have breast cancer. 2) To describe the relationship between levels of 25-hydroxy vitamin D and 1,25-dihydroxyvitamin D and previously described risk factors for breast cancer. 3) To identify and quantify the distribution of vitamin D receptors in tumor samples from the women diagnosed with breast cancer. 4) To identify the relationship between previously described risk factors for breast cancer and blood levels of 25-hydroxy vitamin D and 1,25-dihydroxyvitamin D with vitamin D receptor (+) and vitamin D receptor (-) breast cancers.

BODY

Our previous work includes a validation study to investigate the feasibility of using archived samples of frozen, EDTA-anticoagulated whole blood for the analysis of the active vitamin D metabolite, 1,25-dihydroxyvitamin D. The study was approved by the human subjects research committee of the School of Public Health at the University of North Carolina at Chapel Hill. All assays were done using a radioreceptor assay kit (INCSTAR, Stillwater, Minnesota). The initial phase of this work was supported by an intramural grant from the Lineberger Comprehensive Cancer Center.

Multiple aliquots from pooled plasma in the laboratory of Dr. Gayle Lester were analyzed to determine the inter-assay and intra-assay variability in plasma assays. Whole blood from volunteers was used to assess inter- and intra-assay variability in whole blood assays.

Table 1. Precision of the $1,25(OH)_2D$ assay in pooled plasma and whole blood

	MEDIUM	**N**	**$1,25(OH)_2D$ pg/ml (MEAN ± SD)**	**Coefficient of Variation (%)**
Intra-assay				
	Pooled plasma	11	30.62 ± 1.91	6.2
	Whole blood	2	20.66 ± 1.89	9.1
	Whole blood	2	15.77 ± 2.09	13.2
Inter-assay				
	Pooled plasma	3	27.55 ± 2.68	9.7
	Whole blood	2	19.52 ± 3.19	16.4

Coefficients of variation (CV) were larger for whole blood than for plasma assays; the CV for inter-assay determinations were approximately 70% larger in whole blood (16.4% versus 9.7%), and approximately 80% larger in the intra-assay determinations (11.2% versus 6.2%) (Table 1). Part of the increased variability may be due to the variability in hematocrits of the whole blood samples.

Blood from volunteers was drawn into tubes anticoagulated with EDTA and divided into two aliquots. One aliquot was processed in a fashion identical to the archived

clinical samples; it was frozen as unseparated whole blood at -70^0. The second aliquot was centrifuged at 2000 RPM at room temperature for 10 minutes; the plasma was removed and frozen at -70^0. The samples were retrieved and analyzed after three to seven weeks of frozen storage. Plasma and whole blood were analyzed in the same batches.

The values of the D metabolite were on average 27% lower when determined in whole blood than when determined in plasma from the volunteers (Table 2).

Table 2. Comparison of plasma and whole blood levels of 1,25$(OH)_2$D in nineteen normal volunteers

MEDIUM	N	1,25$(OH)_2$D pg/ml (MEAN + SD)	RANGE (pg/ml)
Plasma	19	31.83 $\pm$ 7.66	18.51 - 48.60
Whole blood	19	23.31 $\pm$ 7.00	14.29 - 44.14

The decrease is probably related to the diluting effect of the hemolyzed red cells on the volume of substrate to be assayed. The red cell mass normally accounts for 30 - 40% of the blood volume; when this mass is not removed, the hemolyzed red cells will dilute the material to be assayed by an amount equal to their volume.

Task 1: Analysis of vitamin D metabolites (0-15 months).

After characterizing the assay in the substrate available to us, we analyzed samples from cases and controls for 1,25-dihydroxyvitamin D. Our vitamin D analyses were done in 13 batches of 40 samples each with an admixture of randomly selected samples consisting of approximately one-third cases and one-third controls from each of the two control groups. We used the radioreceptor assay from INCSTAR (Stillwater, Minnesota) for determination of 1,25$(OH)_2$ D. Four identical samples of pooled plasma were included in each batch to provide quality control through a measure of both intra-assay and inter-assay variability. In addition, controls provided with the assay kits from INCSTAR were analyzed. Laboratory personnel were blinded to the case or control status of the samples. Analyses were carried out from September 15, 1994 through April 4, 1995.

Approximately 11% of the samples have to be redone because of technical difficulties such as low recovery rates; this work is currently in progress.

We have analyzed data from the blood samples of the first 348 of 513 subjects (Table 3).

Table 3. Characteristics of the first 348 study subjects

	Cases (n=103)	Control 1 (n=125)	Control 2 (n=120)
Age, years (SD)	52.8 (12.3)	53.3 (12.4)	52.9 (13.1)
Race			
White	88	102	100
Black	11	19	16
Asian	3	3	2
Hispanic	1	1	2
$1,25(OH)_2$ D (SD) picograms/mL	20.19 (11.31)	22.98 (9.68)	23.31 (9.65)

The groups are comparable in age and racial composition. Our preliminary results are consistent with our research hypothesis. Levels of $1,25(OH)_2$ D are lower in women with breast cancer than in similar women without breast cancer. The difference is greater between the cases and general medical clinic controls (t -2.20, p 0.0293) than between the cases and the breast clinic controls (t -1.98, p 0.049) . The breast clinic controls who are most similar to the breast cancer cases have $1,25(OH)_2$ D levels which are intermediate between the cases and general medical clinic controls.

We have data on 1,25-dihydroxyvitamin D levels by race on 299 subjects (Table 4). At this point we are looking primarily at black-white differences since the number of subjects in other groups is very small. Our data confirm the previous reports of higher blood levels in black subjects than in white subjects.

Table 4. 1,25-dihydroxyvitamin D level, picograms/milliliter by race

Race	N	1,25-dihydroxyvitamin D, mean ± SD
Black	44	23.83 ± 10.71
White	255	21.60 ± 10.50

The work relating to the active metabolite 1,25-dihydroxyvitamin D is on schedule, and the analysis of the remaining samples should be completed within the original time estimate.

The analysis of 25-hydroxyvitamin D in these whole blood samples has not been straight forward. Initial studies were thwarted by the fact that recovery of the metabolite from the whole blood was very low and when these samples were analyzed with the tritium based radioimmunoassay, many samples had non-detectable levels. Since that time, we have begun to use the more sensitive 125 iodine based assay. With this assay, many more of the samples had detectable levels of metabolite, however, the recovery was still low relative to the extraction from plasma. We are currently engaged in studies to determine the variability of the recovery from sample to sample. In the case that it is variable, we will spike each whole blood sample with isotope prior to extraction in order to follow the percentage recovery in a manner similar to that used in the assay of the 1,25-dihydroxyvitamin D metabolite. Initial investigations have shown recoveries to be relatively reproducible in the range of 60-65% of tracer added. This is in contrast to plasma where the recovery is 100%. It is anticipated that these assays with modifications will be underway within the next 2 months.

Task 2: Development of Assay for VDR (0-6 months).

The following methods for immunohistochemical localization of vitamin D receptor in frozen sections of human gut have been used during the past year. In our initial proposal, we planned to use normal and malignant breast tissues for our pilot study. Due to the variability of the presence of the receptor in this tissue, we decided to establish

the methodology using human tissue, fresh frozen and fixed, known to have the VDR. To date, we have had success with obtaining positive immunostaining for the VDR, however, there is high background in these sections in addition to nuclear staining. Efforts are currently underway to reduce the background staining as described below. Once these efforts are successful, methods will be applied to sections of fixed tissue from the same specimens.

Frozen specimens of normal human intestine have been obtained from Dr. M. Koruda at UNC Hospital. The specimens were divided in two with one piece frozen in embedding medium and the other fixed in 10% neutral buffered formalin (the slides have been stored at 4° C for later use) . 5 micron serial sections were cut from the frozen specimens on a cryostat and mounted onto silane coated glass slides (Polysciences, Warrington, PA). Slides were then stored at -80° until staining. All incubations were carried out in a humidified chamber. At the time of immunostaining, slides were fixed for 10 minutes in neutral buffered formalin according to the method of Milde et al (Milde et al, 1989). Following washes with phosphate buffered saline, slides were treated with ice cold methanol and acetone for 3 and 1 minutes. respectively. After rinsing with PBS, sections were blocked by incubation for 15 minutes each with the following agents: 5% normal goat serum, avidin (5mg/ml), and biotin (5mg/ml). Endogenous peroxidase activity was inhibited by incubating sections with 0.3% hydrogen peroxide for 20 minutes. Slides were then incubated with primary antibody, MAb 9A7g at several dilutions for 12 hours at 4°C. After incubation, slides were washed with PBS and incubated for 30 minutes. with biotin-labeled goat anti-rat IgG at room temperature. After two rinses with PBS, slides will be treated with strepavidin-peroxidase for 30 minutes. at room temperature. After two rinses with PBS, staining will be visualized with 0.05% diaminobenzidine with 0.04% hydrogen peroxide solution (DAB) for 6 minutes. or more. Slides were viewed to determine adequate color development and then rinsed, dehydrated

and cleared prior to placement of coverslips. In all cases, controls were run in which primary antibody 9A7g was omitted.

To reduce the background staining due in part to endogenous peroxidases in the tissue, we have tried a streptavidin conjugated to alkaline phosphatase. Unfortunately, this procedure also gave high background staining. At present, experiments are underway using streptavidin conjugated to glucose oxidase. Since this enzyme is not present in high levels in the gut, it should prove to be a better choice for color development. Should this not be successful, efforts will focus on the use of fluorescent dyes conjugated to streptavidin as an alternative.

In preparation for the application of these methods to fixed tissues, we have established a working relationship with Dr. Lester Layfied's laboratory (Department of Pathology , Duke University). In this clinical lab, they have adapted methods for the localization of the estrogen receptor in frozen tissues for use on fixed specimens. Dr. Layfield has offered several protocols for our use and the resources of his laboratory to enable us to successfully adapt the immunohistochemical methods for VDR visualization in frozen specimens for sections of fixed tissue.

Task 3: Detection of VDR's in paraffin-embedded tumor specimens (6-18 months).

Retrieval of tissue blocks is ongoing. As of April 1, 1995 (month 10), we have obtained tumor blocks for 125 cases, 78% of the total. Most of these have been sectioned and are now being reviewed by Dr. Pat Wilson, a pathologist, to localize tumor on the slide. The retrieval segment of the task is on schedule and should be completed within the original time estimate. We have not yet started to analyze the breast tumor specimens with a modified immunohistochemical technique (see Task 2). This segment of the task depends on the successful completion of Task 2; our current work effort is focused on achieving this goal.

Task 4: Data analysis and report writing (15-24 months)

Although the grant has not reached month 15, we have begun analysis of results from the assays of the first 348 study subjects (see Task 1). The preliminary analysis suggests that blood levels of 1,25-dihydroxyvitamin D are lower in women at the time of first diagnosis of breast cancer than in comparable women who do not have breast cancer. This analysis addresses specific aim 1 and supports the research hypothesis. The next phase of the analysis will focus on specific aim 2, the relationship between levels of 25-hydroxy vitamin D and 1,25-dihydroxyvitamin D and previously described risk factors for breast cancer, with particular attention to body mass index.

Regarding the other two points in Task 4, we have a rough draft of a manuscript describing the methods used to assess 1,25-dihydroxyvitamin D assay validity and reliability in whole blood. Part of this work is discussed in the beginning of this section. We anticipate writing a second manuscript on the results of this study as we complete data analysis.

Preparation of the Final Report for the DOD will begin around month 20.

CONCLUSION

Our preliminary data indicate that women with newly diagnosed breast cancer have mean blood levels of 1,25-dihydroxyvitamin D that are 11% lower than those in women without cancer. Among white women, this difference is statistically significant at the 0.05 level. Although our number of black subjects is small, there does not seem to be a similar pattern of difference between cases and controls. Our data confirm previously reported differences between blacks and whites in blood levels of 1,25-dihydroxyvitamin D with black women having levels 12% higher than those of white women.

Future work needs to address the temporal relationship between low levels of vitamin D metabolites and the development of breast cancer. A case-control design study design cannot evaluate the temporal relationship between D metabolites and the development of breast cancer; a prospective study would provide certainty on this temporal relationship. We are currently developing a proposal for a prospective study to investigate the relationship between low levels of vitamin D metabolites and future risk of breast cancer.

Another area for future investigation is the study of black-white differences in vitamin D metabolites and a look at possible racial differences in response to vitamin D supplementation. Furthermore, studying a larger group of black women will clarify the relationship between vitamin D metabolites and risk of breast cancer in these subjects.

The significance of this work relates to the potential for prevention of breast cancer; in addition, there are implications for the development of new therapeutic modalities, and the possibility of better understanding mechanisms of carcinogenesis as we define the role of vitamin D.

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